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(71) Applicant *(for all designated States except US)*: MONASH UNIVERSITY [AU/AU]; Clayton Road, Clayton, VIC 3168 (AU).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: PEURA, Teija [FI/AU]; 2/15 Elata Street, Oakleigh South, VIC 3167 (AU).

(74) Agent: PHILLIPS ORMONDE & FITZPATRICK; 367 Collins Street, Melbourne, VIC 3000 (AU).

(54) Title: A METHOD OF OOCYTE ENUCLEATION AND PRODUCTION OF RECONSTITUTED EMBRYOS

(57) Abstract

The present invention relates to a process for the enucleation of oocytes and the production of cytoplasts and to the use of such cytoplasts and oocytes in a process of nuclear transplantation for the production of nuclear transfer embryos and multiple offspring of genetic similarity. Accordingly, the present invention provides a method for enucleating an oocyte which method includes: providing an oocyte having a polar body, metaphase plate and cytoplasm; subjecting the oocyte to a compound capable of causing attachment of the polar body to the oocyte; and enucleating the oocyte by separating the polar body and a portion of cytoplasm containing the metaphase plate from remaining cytoplasm. In another aspect of the present invention there if provided a method of increasing cytoplasmic volume in an embryonic cell, said method including: providing at least two cytoplast prepared by a method of enucleating an oocyte; providing an embryonic cell; and fusing said cytoplasts with the embryonic cell.

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A METHOD OF OOCYTE ENUCLEATION AND PRODUCTION OF RECONSTITUTED EMBRYOS

The present invention relates to a process for the enucleation of oocytes and the production of cytoplasts and to the use of such cytoplasts and oocytes in a process of nuclear transplantation for the production of nuclear transfer embryos and multiple offspring of genetic similarity.

The benefits of nuclear transplantation are that it enables the rapid increase in numbers of genetically identical animals or progeny having superior production traits. By removal of genetic diversity from animals, and replacing this with common genetic material, characteristics of the animal may be more accurately quantified.

The process of nuclear transplantation is described as the transfer of an intact nucleus from one cell to another which has had its nuclear DNA removed or destroyed. More particularly, the process involves the introduction of a foreign nucleus into the cytoplasm of an enucleated recipient oocyte. Hence the process may be applied to a recipient oocyte which will receive genetic material from a donor nucleus. Foreign genetic material is generally introduced via fusion which reconstitutes the genome of the oocyte. Fusion with the oocytes then results in a reconstituted, transplanted oocyte of known genetic constitution which has the potential of developing into an embryo whose cells may be used in time for nuclear transplantation or the production of embryonic stem cells thereby increasing the potential number of genetically identical embryos. Resulting embryos can be transferred to recipient females to enable the production of primordial germ cells or embryo development to term.

Cloning of mammalian embryos by nuclear transplantation has been used to produce embryos and offspring from a number of species since the first report of successful cloning in sheep by Willadsen in 1986 (Willadsen, 1986).

The methodology used today relies on four main elements;

(1) Enucleation of mature oocytes to produce recipient cytoplasts needed to support early embryonic development

(2) Production and separation of advanced stage embryonic cells serving as a source of clonal material

(3) Fusion of enucleated oocyte and embryonic cell together to create a new, reconstituted embryo

(4) Culture of these embryo clones up to transferable embryo stage.

These techniques are highly skilled, technically demanding, expensive and time consuming techniques. The overall efficiencies of these techniques are relatively low, preventing an effective transfer of technology from experimental laboratories to more commercial environment.

Extrusion of the first polar body is an important marker for oocyte maturation, as it contains the proportion of oocyte chromatin (the genetic material in the oocyte), which has to be removed during the maturation process. After polar body extrusion the remaining chromatin arranges itself to a metaphase plate and arrests at that stage (Metaphase II arrest) until fertilization or activation stimulus causes embryonic development to proceed. However, the first polar body and metaphase plate remain physically close to each other.

In order to ensure that a reconstituted nuclear transfer embryo is indeed derived from the late stage embryonic nucleus which was fused into an enucleated oocyte, it is important to remove all of the oocytes own genetic material prior to cell fusion. If this is not done properly, the resulting embryo may become a parthenogenetic embryo. Mammalian oocytes can readily develop up to morula or blastocyst stage after an artificial activation stimulus without the contribution of the fertilizing sperm (or in case of nuclear transfer embryo, without the contribution complete cell nucleus from a late stage embryo), but as resulting parthenogenetic embryos have only half of the normal number of chromosomes, development can not proceed to live fetus or offspring. Additionally, any oocyte-derived chromatin left in to the reconstituted embryo could cause disturbances in the process of normal chromosome and cell division even without participating directly to embryonic development, thus preventing proper development of nuclear transfer embryo.

Oocyte enucleation is done at the stage of Metaphase II arrest, and the aim is to remove the first polar body and a portion of oocyte cytoplasm

containing the whole metaphase plate and thus the genetic material. Because visual observation of chromatin in bovine oocytes is impossible due to dark, opaque cytoplasm, the position of the polar body can be used to identify the position of the metaphase plate.

In traditional enucleation methods the polar body and some adjacent cytoplasm are aspirated and removed from zona pellucida (shell surrounding oocyte) enclosed oocytes with a micropipette. Another approach is to remove the zona pellucida and bisect the oocyte with a microsurgical blade. Because removal of zona pellucida invariably causes the loss of the polar body, it cannot be used any more as a marker for the metaphase plate, and thus half of the oocyte containing the chromatin has to be identified with other methods, for example by nuclear staining.

If the polar body could be forced to stay in its original position during bisection-enucleation, it could be used to position the metaphase plate, facilitating direct identification and discarding of the oocyte fragment containing chromatin.

The quality of the recipient oocyte is clearly an important factor in successful nuclear transfer. Since all critical maternal factors (such as proteins and messenger-RNAs required for synthesis of new proteins) required for early embryo development are synthetized and stored in the oocyte cytoplasm during oocyte maturation, it would be logical to assume that the amount of these factors in cytoplasm would also play a role in successful cloning. Most studies on nuclear transfer have relied on enucleation by aspiration, where, depending on the skill of the micromanipulator, anything between 5 to 50% of the cytoplasm is removed with a metaphase plate (Westhusin et al. 1992, 1996). Another method of enucleation is to bisect oocytes and discard the chromatin containing half, resulting in loss of 50% of the volume.

Although embryonic development to the blastocyst stage and even live offspring have been achieved with bovine and porcine embryos with reduced cytoplasmic volumes by disaggregating 2, 4 or 8-cell embryos and culturing 1/2, 1/4 and 1/8 blastomeres separately (Saito et al. 1991, Johnson et al. 1995), resulting embryos had fewer cells and resulted in poor pregnancy rates. Only

limited studies have been published about the effects of reduced or increased cytoplasmic volumes on development of nuclear transfer embryos. Greising and co-workers (1994) found increased cleavage and development rates in bovine nuclear transfer embryos, which had received additional cytoplasm as compared to control embryos, which were enucleated by aspiration of zona-enclosed oocytes. However, overall numbers of embryos used for this study were low and no cell number determinations or embryo transfers were performed. Westhusin and co-workers (1996) found no difference in embryonic development rates of bovine nuclear transfer embryos derived from oocytes with 95 or 50% of the original cytoplasmic volume. Nevertheless, mean cell numbers of nuclear transfer blastocysts were higher in 95% volume group, and comparable to control *in vitro* produced embryos. However, in this work only development rates of embryos derived from reduced cytoplasmic volumes were compared and no efforts to increase cytoplasmic volumes were done.

Accordingly, it is an object of the present invention to overcome or at least alleviate one or more of the difficulties and deficiencies of the prior art.

Accordingly, the present invention provides a method for enucleating an oocyte which method includes:

providing an oocyte having a polar body, metaphase plate and cytoplasm;

subjecting the oocyte to a compound capable of causing attachment of the polar body to the oocyte; and

enucleating the oocyte by separating the polar body and a portion of cytoplasm containing the metaphase plate from remaining cytoplasm.

The term "cytoplasm" as used herein means the portion of the oocyte other than the polar body.

Oocytes may be obtained from any source. For example, they may be of bovine, ovine, porcine, murine, amphibian, equine or wild animal origin.

Oocytes may be selected at a particular stage of maturation. The state of maturation may be achieved by *in vitro* culture as described below or the animal may be monitored to provide oocytes at a particular stage of maturation during an ovulation cycle. This may be effected by administration of fertility hormones

and drugs which can synchronise the animal so that the stage of maturation can be determined.

Oocytes selected for enucleation are preferably selected at the stage when the oocytes have extruded the first polar body. Preferably, the oocytes are at the stage of Metaphase II arrest wherein during enucleation the first polar body and a portion of oocyte cytoplasm containing the whole metaphase plate and thus genetic material may be removed. If the oocytes are selected for enucleation after extrusion of the first polar body, it is preferred that they are selected at approximately 20 to 30 hours post maturation, more preferably at approximately 24 hours after initiation of maturation.

The method of the present invention may include the preliminary step of subjecting the oocytes to *in vitro* maturation. Maturation may be controlled by protein synthesis and phosphorylation inhibitors. The preliminary step of subjecting the oocytes to *in vitro* maturation may alter the structure of the oocyte or it may progress the oocyte to a stage where it is more susceptible to enucleation.

The oocytes may be matured *in vitro* by exposure to suitable culture conditions, hormones and/or growth factors. Preferably the oocytes are matured in the presence of a cell culture medium and serum. Preferably the serum includes growth factors and/or gonadotrophic and/or ovarian hormones. The culture medium may be any culture medium capable of sustaining oocytes in culture. Preferably the cell culture medium is TCM199 and the serum is foetal calf serum (FCS).

Where oocytes have been cultured *in vitro* cumulus cells may be removed to provide oocytes at a suitable stage of maturation for enucleation. Cumulus cells may be removed by pipetting or vortexing for example in the presence of 0.5% hyaluronidase.

The polar body may be a first polar body or a second polar body. There may be any number of polar bodies in a oocyte. Preferably there is one polar body. More preferably the polar body is a first polar body.

The compound is selected to attach the polar body to the oocyte such that the polar body remains in the oocyte as an indication of the metaphase

plate. By attaching the polar body to the oocyte it is forced to stay in place despite further manipulations such as enzymatic treatment on the oocyte which may cause loss of the polar body thereby removing any indication of the metaphase plate. The compound may be a compound capable of agglutinating cells. The compound may be a protein or glycoprotein capable of binding or agglutinating carbohydrate. More preferably the compound is a lectin. The lectin may be selected from the group including wheatgerm agglutinin, Concanavalin A, Canavalin A, Ricin, soyabean lectin, lotus seed lectin, phytohemaglutinin (PHA). Most preferably the compound is PHA.

Some lectins such as PHA have been used in nuclear transfer procedures to make embryonic cells to enucleated oocytes before electrofusion, however, this process is simply to keep the cells together.

The concentration of the compound is preferably at a concentration where a polar body is caused to attach to an oocyte. Preferably this would be in the range of 50 to 500 μg/mL. More preferably the concentration is in the range of 100 to 300 μg/mL. Even more preferably the concentration is in the range of 150 to 250 μg/mL. Most preferably the concentration is 200 μg/mL.

When the compound is PHA, the oocyte is preferably subjected to a concentration of 200 μg/mL.

Preferably, the compound is suspended in phosphate buffered saline.

In a further preferred embodiment, the oocytes may be exposed to the compound for a sufficient time to allow attachment of the polar body to the oocyte. Preferably the oocyte is subjected to the compound for a period of up to 60 minutes. More preferably the period is 5 to 30 minutes. Even more preferably the period is 7 to 15 minutes. The oocytes are subjected to the polar body attaching compound at least once. However the oocyte may also be subjected several times to the compound by removing the oocyte or diluting the compound prior to resubjecting the oocyte to the compound. During the removal or dilution step, the oocytes may be transferred to a handling medium, preferably phosphate buffered saline preferably containing cytochalasin B. Preferably the cytochalasin B is 5 to 10 μg/mL, most preferably 7.5 μg/mL.

Enucleation of the oocyte may be conducted by any procedure known to

the skilled addressee. Preferably, enucleation is conducted using a microsurgical blade.

The oocyte may be separated into at least two portions. One portion may include the polar body and a portion of cytoplasm containing the metaphase plate, and the other portion may contain a substantial portion of the cytoplasm of the oocyte. Preferably the cytoplasm removed with the polar body contains the whole metaphase plate.

Enucleation is preferably guided by the polar body which may be used to identify the position of the metaphase plate. Preferably, the oocyte is separated between one portion containing the polar body and the metaphase plate and a portion of the cytoplasm and the other portion being the remaining cytoplasm.

The portion containing the polar body and chromatin is discarded leaving a cytoplast or that portion of the oocyte containing cytoplasm. Preferably, the portion attached to the polar body is 10 to 90% of the total oocyte volume. More preferably the portion may be 20 to 70% of the total oocyte volume. Most preferably the portion may be 40 to 50% of the total oocyte volume.

In a preferred embodiment of the present invention there is provided a method for enucleating oocytes which method includes:

providing an oocyte having a polar body, metaphase plate and cytoplasm;

subjecting the oocyte to a compound capable of causing attachment of the polar body to the oocyte;

removing the zona pellucida from the oocytes to provide a zona-free oocyte containing the polar body, metaphase plate and cytoplasm; and

enucleating the zona-free oocyte by separating the polar body and a portion of cytoplasm containing the metaphase plate from remaining cytoplasm.

Removal of the zona pellucida may be conducted by any means. Generally, in the absence of a compound capable of attaching the polar body to the oocyte, the polar body is lost during removal.

Removal may be by physical or chemical means. Physical means may include the use of a micropipette or by a microsurgical blade. Preferably, removal of the zona is by chemical means. More preferably, removal utilizes an

enzymatic means. Most preferably, the enzyme is a pronase or Protease.

Preferably the concentration of the enzyme used to remove the zona pellucida is in the range of 0 to 5%. More preferably it is in the range of 0.25% to 2%. Even more preferably it is in the range of 0.4% to 0.8%. Most preferably the enzyme concentration is 0.5%.

The oocyte may be treated by the chemical or enzyme for a period of 0 to 60 minutes. More preferably it is treated for a period of 5 to 40 minutes. Even more preferably the period is 10 to 30 minutes.

Optimally, the period required for zona removal should be a period which avoids detrimental effects on the oocyte. Pronase is an effective enzyme that breaks down proteins and a long exposure of oocytes may result in loss of viability of the oocyte.

Treatment with the compound to attach the polar body to the oocyte may affect removal of the zona pellucida. Therefore, dilution of the compound is preferable prior to removal of the zona. An optimal combination of diluting the compound and treatment with an enzyme to remove the zona is adopted.

Preferably, following treatment with the compound, a dilution step is conducted such that the compound is diluted 1:10 to 1:100 and the oocyte is incubated with an enzyme for 5 to 10 minutes for removal of the zona. Most preferably the compound is diluted 1:100 and the oocyte is incubated for 5 minutes to remove the zona.

In another aspect of the present invention, there is provided a cytoplast having a high cytoplasmic volume devoid of a polar body, metaphase plate and substantially free of chromatin.

In a preferred embodiment of the present invention there is provided a cytoplast produced by a method for enucleating an oocyte which method includes:

providing an oocyte having a polar body, metaphase plate and cytoplasm;

subjecting the oocyte to a compound capable of causing attachment of the polar body to the oocyte; and

enucleating the oocyte by separating the polar body and a portion of

cytoplasm containing the metaphase plate from remaining cytoplasm.

In a preferred embodiment of the present invention there is provided a cytoplast produced by a method for enucleating an oocyte, which method includes:

providing an oocyte having a polar body, metaphase plate and cytoplasm;

subjecting the oocyte to a compound capable of causing attachment of the polar body to the oocyte;

removing the zona pellucida from the oocytes to provide a zona-free oocyte containing the polar body metaphase plate and cytoplasm; and

enucleating the zona-free oocyte by separating the polar body and a portion of cytoplasm containing the metaphase plate from remaining cytoplasm.

The term "cytoplast" as used herein means an enucleated oocyte which includes the portion of the oocyte remaining after removal of the attached polar body. The cytoplast may include the cytoplasm and maternal factors required for early embryo development. Preferably the cytoplast is substantially free of genetic material (chromatin).

Accordingly, in another aspect of the present invention there is provided a method for preparing a transplantation embryo which method includes:

providing a cytoplast prepared by a method of enucleating an oocyte said method including the steps of:

providing an oocyte having a polar body, metaphase plate and cytoplasm;

subjecting the oocyte to a compound capable of causing attachment of the polar body to the oocyte; and

enucleating the oocyte by separating the polar body and a portion of cytoplasm containing the metaphase plate from remaining cytoplasm;

providing an embryonic cell;

fusing said cytoplast with the embryonic cell to a produce reconstituted embryo; and

culturing the reconstituted embryo to a transferable embryo stage.

In a preferred aspect, there is provided a method for preparing a

transplantation embryo which method includes:

providing a cytoplast prepared by a method of enucleating an oocyte said method including the steps of:

providing an oocyte having a polar body, metaphase plate and cytoplasm;

subjecting the oocyte to a compound capable of causing attachment of the polar body to the oocyte;

removing the zona pellucida from the oocytes to provide a zona-free oocyte containing the polar bodym metaphase plate and cytoplasm;

enucleating the zona-free oocyte by separating the polar body and a portion of cytoplasm containing the metaphase plate from remaining cytoplasm;

Culturing of the reconstituted embryo may be conducted by any means available to the skilled addressee.

The embryos so produced by this method may be used to produce genetically identical or similar animals by transplantation into a recipient female, preferably a synchronised female. Preferably, the recipient female is synchronised using fertility drugs, steroids or prostaglandins.

In another aspect of the present invention there is provided a method of increasing cytoplasmic volume in an embryonic cell said method including:

providing at least two cytoplast prepared by a method of enucleating an oocyte;

providing an embryonic cell; and

fusing said cytoplasts with the embryonic cell.

The cytoplast may be prepared by any method which results in enucleation of the oocyte. Many of the methods are known by the skilled addressee and include dissection or bisection of the oocyte using a metal blade, aspiration and centrifugation. Preferably, the cytoplast are prepared by bisection using a metal blade.

Most preferably, the cytoplast is prepared by a method of enucleating an oocyte as described herein.

At least two cytoplasts are fused to the embryonic cell, however, more cytoplasts may be fused to the embryonic cell. Preferably three components are

fused, that is, two cytoplasts and one embryonic cell.

Embryonic cells may be obtained from any source. For example, they may be of bovine, ovine, porcine, murine, amphibian, equine or wild animal origin.

Embryonic cells may be selected at a particular stage of development. The stage of development may be achieved by *in vitro* culture as described below or the animal may be monitored to provide embryonic cells at a particular stage of development. This may be effected by administration of fertility hormones and drugs which can synchronise the animal so that the stage of development can be determined.

The process of the present invention may include the preliminary step of subjecting the embryonic cell to *in vitro* culture. Development may be controlled by protein synthesis and phosphorylation inhibitors. The preliminary step of subjecting the embryonic cells to *in vitro* culture may alter the structure of the embryonic cell or it may progress the embryonic cell to a stage where it is more susceptible to fusion.

The embryonic cells may be cultured *in vitro* by exposure to suitable culture conditions, hormones and/or growth factors. Preferably the embryonic cells are matured in the presence of the cell culture medium and serum. Preferably the serum includes growth factors and/or gonadotrophic and/or ovarian hormones, The culture medium may be any culture medium capable of sustaining embryonic cells in culture. Preferably the cell culture medium is TCM199 and the serum is foetal calf serum (FCS).

Fusion of the cytoplast and the embryonic cell may be conducted by any method familiar to those skilled in the art. Preferably the process is conducted in an electrofusion chamber with at least one pulse.

The fusion of the cytoplast with the embryonic cell may increase the cytoplasmic volume by approximately 100 to 150%, preferably 100 to 120% of the normal oocyte volume for a reconstituted embryo.

The embryonic cells may be at least any stage. Preferably, the cells are at an advanced stage in development such as to serve as a source of clonal material.

Accordingly in another aspect, the present invention provides an embryonic cell having an increased cytoplasmic volume.

In another aspect there is provided a method for preparing a transplantation embryo which method includes:

providing at least two cytoplast prepared by a method of enucleating an oocyte;

providing an embryonic cell;

fusing said cytoplast with the embryonic to produce a reconstituted embryo; and

culturing the reconstituted embryo to a transferable embryo stage.

Culturing of the reconstituted embryo may be conducted by any means available to the skilled addressee.

In a further aspect of the present invention, there is provided a transplantation embryo prepared according to the method described above.

The embryos so produced by this method may be used to produce genetically identical or similar animals by transplantation into a recipient female, preferably a synchronised female. Preferably, the recipient female is synchronised using fertility drugs, steroids or prostaglandins.

The modifications described above have been aimed at improving overall development rates of nuclear transfer embryos, but also at making methods easier to apply in an environment outside research laboratories, i.e. by reducing the need for specialised equipment and highly skilled person to perform the techniques. The improvements described in this document concentrate on oocyte enucleation and on cell fusion.

IN THE FIGURES:

Figure 1 shows a schematic presentation of mature bovine oocyte with the first polar body and a metaphase plate. The vertical lines represent splitting of oocytes into various sized fractions.

REFERENCES:

Greising, T., Monaghan, P., Healy, A., Gordon, A., Gallagher, M., Wahid, H. & Gordon, I. (1994). The influence of cytoplasmic composition and quantity of cytoplasm in recipient oocytes on the developmental capacity of bovine nuclear transfer embryos. Theriogenology 41:208.

Johnson, W., Loskutoff, N., Plante, Y. & Betteridge, K. (1995). Production of four identical calves by separation of blastomeres from an in vitro derived four cell embryos. Vet. Rec. 137:15-16.

Peura, T.T., Wild, S.P. & Trounson, A. (1997). The effect of cytoplasmic volume on development of bovine nuclear transfer embryos derived from in vivo donor embryos. Theriogenolog
y (in press)

Saito, S. & Niemann, H. (1991). Effects of extracellular matrices and growth factors on the development of isolated porcine blastomeres. Biol. Reprod. 44:927-936.

Westhusin, M., Levanduski, M., Scarborough, R., Looney, C. & Bondioli, K. (1992). Viable embryos and normal calves after nuclear transfer into Hoechst stained enucleated demi-oocytes of cows. J. Reprod. Fert. 95:475-480.

Westhusin, M., Collas, P., Marek, D., Sullivan, E., Stepp, P., Pryor, J. & Barnes, F. (1996). Reducing the amount of cytopplasm available for early embryonic development decreases the quality but not quantity of embryos produced by in vitro fertilization and nuclear transplantation. Theriogenology 46:243-252.

Willadsen, S. M. (1986). Nuclear transplantation in sheep embryos. Nature 320:63-65.

The present invention will now be more fully described with reference to the following examples. It should be understood however that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

Example 1

Enucleation of mature oocytes to produce recipient cytoplasts need to support early embryonic development

- oocyte enucleation by bisection and the use of PHA facilitating identification of cytoplasts

Immature bovine oocytes are aspirated form 2-10 mm follicles of abattoir derived ovaries and washed twice in Tissue Culture Medium 199 supplemented with 4 mg/ml Bovine Serum Albumin and 17.5 mM HEPES-buffer. Cumulus cell ooctye complexes are matured at 39°C in an atmosphere of 5% CO_2 in air in Falcon-dishes, 50 oocytes per 800 µl. Maturation medium is a modified TCM199 solution containing 10% Fetal Calf Serum (FCS) and 0.005 IU/ml of hormones LH and FSH. 21 to 24 hours after maturation oocytes are vortexed in 100 µl of handling medium (HEPES-buffered TCM199 + 10% FCS) in an Eppendorf tube for 2 minutes to remove cumulus cells. Matured oocytes for further processing are then selected visually under the stereomicroscope according to the presence of the first polar body.

The selected mature oocytes are transferred into phytohemagglutinin-solution (200 µg/ml in phosphate buffered saline). This is done by picking the oocytes into the glass pipette in a volume of 5 µl and dispensing this into a 45-µl drop of PHA. Thus the dilution of handling medium carried over with oocytes is two times 1:10-dilution, i.e., total of 1:100-dilution. Oocytes are then incubated in the second PHA-drop for 5 minutes, after which they are either transferred into handling medium drop or abundance of handling medium (>200 µl) is added into PHA-drop.

After PHA-incubation zona pellucida of oocytes is removed by 10-30 minutes incubation in 0.5% pronase-enzyme in handling medium. Batches of approx. 20-40 zona-free oocytes are then transferred to 40-µl bisection drops under oil consisting of handling medium supplemented with 7.5 µg/ml cytochalasin B. Oocytes are bisected using free hand into two halves with a metal blade, positioning oocytes so that the half still attached to the polar body is 40 to 50% of the total oocyte volume. This half is then removed and discarded and remaining, chromatin-free half used as a recipient in nuclear transfer.

Phytohemagglutinin (PHA) is a lectin isolated from Phascolus Vulgasris (Red Kidney Bean). Generally lectins are proteins or glycoproteins that agglutinate cells together through their carbohydrate binding properties. The concentration of PHA used in the process is 200 µg/ml, prepared in protein-free phosphate buffered saline (PBS).

Example 2

Attachment of polar bodies to mature bovine oocytes after PHA-incubation

Introduction

Phytohemagglutinin (PHA) is a lectin, that agglutinates cells (e.g. erythrocytes) together through its carbohydrate binding properties. Thus it could be envisaged to be used to attach also other types of cells together.

Aims

(1) To test whether incubation of mature bovine oocytes in phytohemagglutinin in a concentration used in literature to attach embryonic cells into the oocyte would firmly attach first polar bodies to the oocytes.

(2) To test whether highly asymmetrical splitting (resulting in fragments of approximately 30 and 70% of the total oocyte volume) of polar body containing oocytes would result in efficient enucleation.

Materials and methods

Immature bovine oocytes were aspirated from 2-10 mm follicles of abattoir derived ovaries and washed twice in Tissue Culture Medium 199 supplemented with 4 mg/ml Bovine Serum Albumin and 17.5 mM HEPES-buffer. Cumulus cell oocyte complexes were matured at 39 C in an atmosphere of 5% CO_2 in air in Falcon-dishes, 50 oocytes per 800 µl. Maturation medium was a modified TCM199 solution containing 10% Fetal Calf Serum (FCS) and 0.005 IU/ml of hormones LH and FSH. 21 to 24 hours after maturation oocytes were vortexed in 100 µl of handling medium (HEPES-buffered TCM199 + 10% FCS) in an Eppendorf tube for 2 minutes to remove cumulus cells. Matured oocytes for further processing were then selected visually under the stereomicroscope according to the presence of the first polar body.

Selected mature oocytes were then transferred into approx. 30-40 µl drops of phytohemagglutinin-solution (200 µg/ml in Phosphate Buffered Saline) and incubated for 10 minutes, after which they were transferred into 0.5% pronase drop through one wash in handling medium drop. The dissolving of zona pellucidae was observed every few minutes and zona-free oocytes subsequently transferred to handling drops. Batches of approx. 20 zona-free oocytes were pre-incubated for 10 to 15 minutes in TCM199 supplemented with 10% FCS, 7.5 µg/ml cytochalasin B and 5 µg/ml Hoechst 33342 before transfer to bisection drops under oil consisting of handling medium supplemented with 7.5 µg|ml cytochalasin B. At the time of bisection the number of oocytes still containing polar body attached to them was observed.

Oocytes were splitted asymmetrically into fragments representing approximately 30 and 70% of the total oocyte volume using a metal blade and a free hand. The smaller fragment still attached to the polar body was subsequently transferred into adjacent handling medium drop and examined for the presence of chromatin with Nikon Diaphot microscope equipped with an epifluorescence. Oocyte fragments were irradiated with UV for no longer than 1-2 sec, and based on observations on the presence of chromatin, percentages

of successful enucleations were calculated.

Results

The proportion of oocytes still retaining polar bodies after PHA-incubation and zona pellucida removal is shown in Table 1. Results from three separate experimental replicates and the pooled results of all are shown.

Table 1. Presence of the first polar body after PHA-incubation

	Oocytes treated with PHA and checked for polar body (n)	Polar body still attached
Replic. I	118	113 (95.8%)
Replic. II	95	92 (96.8%)
Replic. III	76	72 (94.7%)
Total	289	277 (95.8%)

The proportion of oocytes successfully enucleated after highly asymmetrical oocyte splitting is shown in Table 2. Results from three separate experimental replicates and the pooled results of all are shown.

Table 2. The efficiency of enucleation based on polar body position

	Oocytes splitted (n)	Oocytes successfully enucleated
Replic. I	110	98 (89.1%)
Replic. II	84	76 (90.5%)
Replic. III	67	59 (88.1%)
total	261	233 (89.3%)

Discussion and conclusions

The results of these experiments show that PHA-incubation results in attachment of first polar bodies in bovine mature oocytes in approximately 96% of cases. Without PHA-incubation practically 100% of polar bodies would be

lost, as they are not part of the oocyte any more but extruded, discarded fragments. The few cases where polar bodies were lost despite of PHA-incubation may be contributed to the rough handling, resulting in mechanical removal of polar body, to the situation where the oocyte might not have possessed the first polar body to begin with, but was mistakenly selected to be processed (as selection of oocytes with first polar bodies is based on visual observation, it may not always be 100% accurate) or some unexplained, unidentified factor. However, this level of attachment can be considered satisfactory for further treatment of oocytes.

The aim of this experiment was to find out the efficiency of enucleation based only on position of the first polar body in cases where a minimum amount of cytoplasm was removed. At the time of these experiments the possibility of using two cytoplasts per nuclear transfer embryo was not seriously considered, and the aim was to enucleate oocytes with as little loss of cytoplasm as possible and use resulting single cytoplasts for cell fusions.

The results show that enucleation was indeed successful in almost 90% of the cases. This figure is lower, but not drastically lower than the successful enucleation rate using the method of symmetrical oocyte bisection and subsequent checking of one half of each oocyte with UV to confirm the presence of chromatin (665/697 (95%) successfully enucleated as confirmed by fixing and orcein-staining of demi-oocytes, T. Peura, unpublished observations).

Thus the efficiency of enucleation can be considered quite good. Since the position of metaphase plate in relation to polar body is not expected to vary a lot, the removal of even larger fragment of cytoplasm adjacent to polar body can be confidently expected to increase the rate of enucleation even further (Fig. 1).

Fig. 1. Schematic presentation of mature bovine oocyte with the first polar body and a metaphase plate. The vertical lines represent splitting of oocytes into

various sized fractions.

Example 3:

Optimization of PHA-incubation procedures to ensure consistent outcome of the treatment aimed at attaching first polar bodies to mature bovine oocytes

Introduction

In previous experiments it was noticed that PHA-incubation gave sometimes inconsistent results. Occasionally after PHA-incubation zona pellucidae were lost very rapidly in pronase-enzyme treatment and usually in these cases a large proportion of oocytes lost their polar bodies. On the other hand, occasionally zona pellucidae dissolved very slowly, resulting in slight oocyte degeneration due to required prolonged incubation in pronase. This inconsistency was found to be disturbing from an experimental point of view.

Aims

To test different dilution effects and different incubation times of in vitro-matured bovine oocytes in phytohemagglutinin containing medium in order to find conditions providing reliable, consistent effects.

Materials and methods

Abattoir-derived bovine oocytes were collected and matured in vitro as described in this document in Experiment 1. Phytohemagglutinin-solution was prepared as usual, 200 µg/ml in PBS, and 45-µl drops were prepared under oil. After selection of mature oocytes containing the first polar body, groups of these oocytes were transferred into PHA-drops in 5-µl volume. Different dilutions were achieved by transferring oocytes either directly into one drop or by serially transferring them through two or three drops, always in 5-µl volume. Oocytes from different dilution groups were incubated in their final drops for either 5 or 10 minutes before transferring them through one wash drop to 0.5% pronase drop for zona pellucida removal. Thus experimental groups were as follows:

Group:	Dilution:	Incubation time:
1.	1:10	5 min
2.	1:100 (1:10+1:10)	5 min
3.	1:1000 (1:10+1:10+1:10)	5 min
4.	1:10	10 min
5.	1:100 (1:10+1:10)	10 min
6.	1:1000 (1:10+1:10+1:10)	10 min

After PHA-treatment the time of zona dissolving as well as the proportion of oocytes retaining their polar body after zona removal was observed.

Results

The times required for dissolving of zonae and the proportion of oocytes retaining their polar body after zona removal are shown in Table 3.

Table 3. Dissolving of zonae and polar body attachment after different PHA-treatments

Dilution	Incubation time	Incubation in pronase	Zonae removed(%)	Pb attached (%)
1:10	5 min	10 min	15/15 (100)	
1:100	5 min	20 min	17/17 (100)	44/46 (96)
1:1000	5 min	1 h	16/16 (100)	
1:10	10 min	15-25 min	29/29 (100)	29/29 (100)
1:100	10 min	1 h 20 min	26/29 (90)	22/24 (92)
1:1000	10 min	1 h 20 min	19/20 (95)	18/19 (95)

Discussion and conclusions

Phytohemagglutinin agglutinates cells together through its carbohydrate binding properties. Zona pellucida of mammalian oocytes consists mainly of three different glycoproteins, and based on our observations incubation of zona enclosed oocytes in PHA results in some degree of "zona hardening", making them more resistant to dissolving in pronase.

The optimal PHA-treatment requires the shortest possible time required for zona removal in order to avoid possible detrimental effects of prolonged pronase incubation. After all, pronase is an effective enzyme that breaks down proteins and a long exposure of oocytes for it may result in loss of viability. On the other hand, swift zona removal usually reflects inadequate incubation in PHA to ensure that polar bodies stay attached to oocytes.

These results show that the dilution of 1:10 or 1:100 and incubation time of 5 minutes as well as A dilution of 1:10 and incubation time of 10 minutes gave the best results of PHA-treatment: the shortest pronase incubation required for

zona pellucida removal, but still good attachment of polar bodies to oocytes. Based on these results the treatment consisting of 1:100 dilution and 5 min incubation has been selected as a routine procedure in our nuclear transfer experiments.

Example 4

Development of bovine nuclear transfer embryos using cytoplasts produced by PHA-enucleation method

Introduction

As no published reports were available to show the effects of PHA-enucleation on subsequent embryonic development of bovine nuclear transfer embryos, it was necessary to conduct experiments to confirm that this method of enucleation does not have adverse effects on embryo development.

Aims

To observe the development of bovine nuclear transfer embryos using cytoplasts produced by PHA-enucleation method.

These experiments are a part of another set of experiments studying the effect of reduced and increased cytoplasmic volumes on development of nuclear transfer embryos.

Materials and methods

Cytoplasts were produced as described in this document in Experiment 1, resulting in cytoplasts consisting approximately 75% of the original oocyte volume.

Donor embryos were either in vitro-produced, in vivo-fresh or in vivo-frozen-thawed. Zona pellucida of donor embryos was removed with 0.5% pronase, followed by 10-20 min incubation and disaggregation into individual blastomeres in $Ca^{++}Mg^{++}$-free TALP-HEPES containing 5 µg/ml cytochalasin B.

Blastomeres from each donor embryo were equally distributed to experimental groups. Group 1 consisted of blastomeres fused with single cytoplasts using one pulse (500 V/cm) and Group 2 of two cytoplasts fused with one blastomere using two consecutive fusion steps (cytoplast fusion 800 V/cm followed by blastomere fusion 500 V/cm). All cytoplasts and blastomere handlings were performed in HEPES-buffered TCM199 + 10% FCS, except the fusions which were performed in 0.25 M sucrose. For cell fusions cytoplasts and blastomeres were first equilibriated in fusion medium for few minutes, followed by transfer into an electrofusion chamber. The chamber consisted of parallel wires with a diameter of 0.5 mm and a separation of 0.5 mm. Initial alignment of cells was achieved with an AC pulse of 8 volts and 500 kHZ for 5-10 sec and the actual fusion pulse consisted of a single DC pulse of 99.9 µsec with an amplitude of 500 or 800 V/cm. Cell pairs were allowed to recover in handling medium for 20-40 min before transferring successfully fused pairs into culture drops.

All reconstituted embryos were cultured in microdrops of modified SOF-medium supplemented with 32 mg/ml BSA. Embryo cleavage and cell numbers were observed 2 days after fusion and development to morulae and blastocysts 7 days after fusion. Data was analysed with chi-square (fusion, cleavage, embryonic development) and analysis of variance (cell numbers) using SPSS for Windows-statistical program.

Results

Development of bovine nuclear transfer embryos to morulae and blastocysts in this experiment was 22.5 and 20.0% in Groups 1 and 2, respectively. Neither fusion and cleavage rates nor development rates differed between clones consisting 75 or 150% of the original oocyte volume (Table 4). Embryonic cell numbers of individual bovine nuclear transfer embryos originating from 75 or 150% cytoplasmic volume two and seven days post fusion is shown in Table 5. Significant differences between groups can be observed two days after fusion, but due to large ariation in blastocyst cell numbers, this difference is lost by Day seven.

Table 4. Development rates to morulae and blastocyst at Day 7 of bovine nuclear transfer embryos originating from 75 or 150% cytoplasmic volume[a]

Exp.group	Fusion (%)	Cleavage (%)	M+B (%)	B (%)
1	89/106 (84.0)	74/89 (83.1)	20/89 (22.5)	15/89 (16.9)
2	85/103 (82.5)	70/85 (82.4)	17/85 (20.0)	16/85 (18.8)

M=morulae, B=blastocysts

[a] differences not statistically different between groups

Table 5. Embryonic cell numbers of individual bovine nuclear transfer embryos originating from 75 or 150% cytoplasmic volume 2 and 7 days post fusion

Exp. group	N	Mean cell no.±S.D. 2 dPF (range)	N	Mean cell no.± S.D. 7 dPF (range)
1	47	4.3 ± 2.0^{a} (2-9)	15	78.2 ± 31.7 (39-143)
2	68	5.4 ± 2.8^{b} (2-11)	16	100.3 ± 44.9 (46-194)

[a,b] different superscripts within a column differ statistically ($p<0.05$)

Discussion and conclusions

Development of bovine nuclear transfer embryos to transferable morulae and blastocyst using cytoplasts produced by PHA-enucleation method is comparable to development rates achieved with cytoplasts produced by simple bisection method, when more than half of oocytes original cytoplasmic volume

is used for reconstituted embryos (e.g., 21 and 25% development rate from double cytoplast fusions, Peura et al. 1997). The development rates are also comparable to other published studies on bovine nuclear transfer embryos, e.g., 17% development rate after in vivo-culture (Westhusin et al. 1996).

Although direct comparison of development rates of nuclear transfer embryos using cytoplasts produced with different methods were not done, it is safe to conclude that PHA-enucleation does not have adverse effects on subsequent development of embryos.

Example 5

Fusion of enucleated oocyte and embryonic cell together to create a new, reconstituted embryo

- use of double cytoplast to increase developmental potential of nuclear transfer embryos

Zona pellucida of donor embryos is removed with 0.5% pronase, followed by 10-20 min incubation and disaggregation into individual blastomeres in Ca++Mg++-free HEPES-buffered medium containing 5 μg/ml cytochalasin B. All subsequent cytoplasts and blastomere handlings are performed in TCM 199 plus 10% FCS and HEPES. For cell fusions cytoplasts and blastomeres are first equilibrated in fusion medium (0.25 M sucrose) for few minutes, followed by transfer into an electrofusion chamber. The chamber consists of parallel wires with a diameter of 0.5 mm and a separation of 0.5 mm. Initial alignment of cells is achieved with an AC pulse of 8 volts and 500 kHZ for 5-10 sec and the actual fusion pulse consists of a single DC pulse of 99.9 μsec with an amplitude of 500-800 V/cm. Cell pairs are allowed to recover in handling medium for 20-40 min before transferring successfully fused pairs into culture drops.

Increase in cytoplasmic volume is performed simply by fusing two cytoplasts and one blastomere together simultaneously using one pulse, thus providing reconstituted embryos and the final cytoplasmic volume of 100-150% of the original oocyte volume.

Example 6

Development of bovine nuclear transfer embryos derived from single or double cytoplasts

Introduction

Because of the important maternal factors stored in oocyte cytoplasm and utilized for early embryonic development, the loss of cytoplasm may have effect on subsequent embryonic development. This has shown to be the case normal, non-nuclear transfer embryos and to some degree also on nuclear transfer embryos.

Aims

To compare the developmental potential of bovine nuclear transfer embryos with reduced, excess and normal amount of cytoplasm, i.e., with reduced, excess and normal embryonic volume, i.e., with increased, reduced and normal nucleocytoplasmic ratio.

Materials and methods

Cytoplasts were produced by removing zona pellucidae of in vitro-matured oocytes with the first polar body by incubation in 0.5% pronase, followed by pre-incubation in TCM199 supplemented with 10% FCS, 7.5 µg/ml cytochalasin B and 5 µg/ml Hoechst 33342 before transfer to bisection drops under oil consisting of handling medium supplemented with 7.5 µg|ml cytochalasin B. Oocytes were bisected using free hand into equal halves with a metal blade. One demi-oocyte was subsequently transferred into adjacent handling medium drop and examined for the presence of chromatin with Nikon Diaphot microscopy equipped with an epifluorescence. Based on this examination only

truly enucleated demi-oocytes (cytoplasts) were selected for further treatment. After enucleations all cytoplasts were activated in 38 µM Ca++-Ionophore in protein free TCM199 for 5 min followed by 4 hour incubation in 2 mM 6-DMAP in TCM199 + 10% FCS and transfer to TCM199 + 10% FCS until used for cell fusions 5-7 h after activation.

Donor embryos were either in vitro-produced, in vivo-fresh or in vivo-frozen-thawed. Zona pellucida of donor embryos was removed with 0.5% pronase, followed by 10-20 min incubation and disaggregation into individual blastomeres in $Ca^{++}Mg^{++}$-free TALP-HEPES containing 5 µg/ml cytochalasin B. Blastomeres from each donor embryo were equally distributed to experimental groups. Group 1 consisted of blastomeres fused with single cytoplasts using one pulse (500 V/cm) and Group 2 of two cytoplasts fused with one blastomere using two consecutive fusion steps (cytoplast fusion 800 V/cm followed by blastomere fusion 500 V/cm). All cytoplasts and blastomere handlings were performed in HEPES-buffered TCM199 + 10% FCS, except the fusions which were performed in 0.25 M sucrose. For cell fusions cytoplasts and blastomeres were first equilibrated in fusion medium for few minutes, followed by transfer into an electrofusion chamber. The chamber consisted of parallel wires with a diameter of 0.5 mm and a separation of 0.5 mm. Initial alignment of cells was achieved with an AC pulse of 8 volts and 500 kHZ for 5-10 sec and the actual fusion pulse consisted of a single DC pulse of 99.9 µsec with an amplitude of 500 or 800 V/cm. Cell pairs were allowed to recover in handling medium for 20-40 min before transferring successfully fused pairs into culture drops.

All reconstituted embryos were cultured in microdrops of modified SOF-medium supplemented with 32 mg/ml BSA. Embryo cleavage and cell numbers were observed 2 days after fusion and development to morulae and blastocysts 7 days after fusion. Data was analysed with chi-square (fusion, cleavage, embryonic development) and analysis of variance (cell numbers) using SPSS for Windows-statistical program.

This experiment consisted of three experimental groups; Group 1 consisted of blastomeres fused with single cytoplasts with one pulse and Group 2 of blastomeres fused with two cytoplasts simultaneously with one pulse. In Group 3, two cytoplast were first fused together using one pulse and after recovery time of 30-60 min resulting double cytoplasts were fused again with blastomeres using another pulse. Separate experiments were performed with in vivo and in vitro produced donor embryos.

Fusion and cleavage rates, development to morulae and blastocysts 7 days after fusion and cell numbers 2 and 7 days after fusion were observed. Data was analysed with chi-square (fusion, cleavage, embryonic development) and analysis of variance (cell numbers) using SPSS for Windows-statistical program.

Results

Fusion and cleavage rates and development to morulae and blastocysts 7 days after fusion of in vitro-derived nuclear transfer embryos are shown in Table 5. In Table 6. the same data from in vivo-derived embryos is shown. Mean cell numbers two and seven days after fusion of in vitro-derived embryos is shown in Table 7.

Table 5. Development rates of individual in vitro-derived bovine nuclear transfer embryos originating from 50 or 100% cytoplasmic volume to morulae and blastocyst at Day 7[a]

Group	Fusion-%	Cleavage-%	M+B (%)	B (%)
1	88.2	89.1	28/142 (19.7)[a]	25/142 (17.6)[a]
2	90.3	84.7	50/154 (32.5)[b]	45/154 (29.2)[b]
3	87.8	89.9	32/142 (22.5)[b]	27/142 (19.0)[ab]

M=morulae, B=blastocysts

[a] fusion rate of cytoplast with each other in 2x2 group was 93.8%

[a,b] different superscripts within a column differ statistically ($p<0.05$)

Table 6. Development rates of individual in vivo-derived bovine nuclear transfer embryos originating from 50 or 100% cytoplasmic volume to morulae and blastocyst at Day 7[a]

Group	Fusion-%	Cleavage-%	M+B (%)	B (%)
1	85	88	3/28 (6)[a]	2/48 (4)[a]
2	91	82	9/43 (21)[b]	8/43 (19)[b]
3	88	89	12/48 (25)[b]	12/48 (25)[b]

M=morulae, B=blastocytes

[a] fusion rate of cytoplast with each other in 2x2 group was 96%

[a,b] different superscripts within a column differ statistically ($p<0.05$)

Table 7. Embryonic cell numbers of individual in vitro-derived bovine nuclear transfer embryos originating from 50 or 100% cytoplasmic volume 2 and 7 days post fusion

Exp. group	N	Mean cell no.±S.D. 2 dPF (range)	N	Mean cell no.± S.D. 7 dPF (range)
1	147	4.0 ± 1.6[a] (2-8)	23	70.9 ± 25.4 (39-127)
2	150	5.6 ± 2.2[b] (2-10)	41	88.5 ± 43.5 (29-204)
3	143	5.3 ± 2.2[b] (2-13)	25	86.1 ± 42.7 (25-168)

[a,b] different superscripts within a column differ statistically ($p<0.05$)

Discussion and conclusions

Embryos derived from double cytoplast fusions and one-step electrofusion supported embryonic development clearly better than embryos derived from single cytoplast fusions or even embryos derived from double cytoplast fusions, but produced by two-step electrofusion. In contrast to the work by Westhusin and co-workers (1996), who noted no differences in development rates of nuclear transfer embryos with different cytoplasmic volumes, in this study the difference was clear.

Mean embryonic cell numbers two days after fusion were in embryos resulting from increased cytoplasmic volumes. Mean embryonic cell numbers at blastocyst stage were also higher in embryos resulting from increased cytoplasmic volumes, although due the large variation the difference was not statistically different.

Overall, results of this study support observations that heavily reduced cytoplasmic volumes (by half) don't support embryonic development as well as normal cytoplasmic volumes. The method by which double cytoplast are

produced plays also role in the outcome, one-step fusion method producing better results than two-step procedure, irrespective of the cytoplasmic volume. Best development was achieved with embryos resulting from the normal amount (100% of the original oocyte) of cytoplasm, produced by one-step fusion method.

Example 7

The effect of cytoplasmic volume on development of bovine nuclear transfer embryo derived from in vivo donor embryos

Irrespective of the enucleation method used in cloning, oocyte volume is often reduced by 30-50%. In this experiment we studied whether cytoplasmic volume has en effect on development of nuclear transfer bovine embryos derived from in vivo donor embryos.

Slaugherhouse oocytes were matured in vitro for 22 hours, after which mature oocytes were enucleated by removing zonae in 0.5% pronase, staining with 5 μg/ml Hoeschst 33342 and bisecting into two equal halves in medium containing 7.5 μg/ml cytochalasin, Cytoplasts were assessed for the presence of chromatin with fluorescence microscopy and activated by 5 min incubation in 38 μM Ca^{++}-Ionophore and 4 hour incubation in 2 mM 6-DMAP. Donor embryos were flushed from superovulated cows 4.5 days after insemination, their zonae removed by pronase and embryos disaggregated in $Ca^{++}Mg^{++}$-free medium containing 5 μg/ml cytochalasin. Cytoplasts and blastomers were aligned in 0.25 M sucrose with an AC pulse of 8 vlts and 500 kHZ for 5-10 sec and fused with a single DC pulse of 99.9 μsec and either 1000 V/cm (cytoplast fusions) or 625 V/cm (cytoplast-blastomere fusions). Group 1 consisted of cytoplasts simultaneously using one pulse and group 3 of two cytoplasts, first fused together each donor embryo were equally divided between treatments. Fused cell pairs were cultured in modified synthetic oviduct fluid medium (SOF) supplemented with 32 mg/ml BSA (Gardner et al. 1994, Biol. Reprod. 50:390) at 39°C, in 7% O_2, 5% CO_2 and 88% N_2 for seven days. In the first experiment

(two replicates) three cleaved embryos from same group were aggregated together and cultured as aggregates, whereas in the second experiment (three replicates) embryos were cultured individually. Fusion and cleavage rates, cell numbers two days after fusion, development to morulae and blastocysts and pregnancy rates after embryo transfer were recorded. Differences in cell numbers and development rates between groups were compared by analysis of variance and chi-square, respectively.

Fusion rates were 85, 91 and 88% for groups 1, 2 and 3, respectively. Cleavage rates were 88,92 and 89%, respectively. Neither rates differed statistically between groups.

Table 8. Results of embryo cultures and transfers of nuclear transfer embryos

Exp. group	Culture mode	Blastocysts at day 7/fused		No. of embryos transf./recipients	Pregnant at Day 40
1. (single cytoplast, 1 pulse)	Aggregate	7/10	(70%)	7/3	1/3
	Individual	2/48	(4%)[a]	3/1	1/1
2. (double cytoplasts, 1 pulse)	Aggregate	6/11	(55%)	6/2	0/1
	Individual	8/43	(19%)[b]	9/3	0/3
3. (double cytoplasts, 2 pulses)	Aggregate	7/11	(64%)	5/2	2/2
	Individual	12/48	(25%)[b]	11/3	2/3

[ab] values within a culture mode with a different superscript significantly different ($p<0.05$)

The development rates of embryo aggregates were very high, but not statistically different between groups. Since it is difficult to assess how many embryos participated in development, the true development rate of clones cold be anything between 20 and 70%. However, in individual cultures double cytoplast-clones developed significantly better. Mean cell number ($\pm$ S.D.) of reconstituted embryos at day 2 were 4.0$\pm$ 1.5, 4.7$\pm$2.0 and 4.9$\pm$2.2 for groups 1, 2 and 3 respectively (group 1 differed statistically ($p=0.012$) from groups 2 and 3). Blastocyst cell number determinations were not done, as most of the embryos were transferred to recipients, but further studies are need to

determine whether the early differences in cell numbers are also detectable later.

We conclude that increasing the cytoplasmic volume of reconstituted embryos is beneficial for further embryonic development at least in vitro up to the blastocyst stage, and that this beneficial effect can be observed as early as two day after cell fusion. (Supported by Genetics Australia Co-op. Soc. Ltd.)

Example 8

Post transfer viability of bovine nuclear transfer embryos cultured as aggregates or as individual clones

Pregnancy rates achieved with embryos subjected to in vitro manipulation such as nuclear transfer are generally low and the frequency of early abortion high. Since most manipulation procedures result in reduced cell numbers, this experiment was conducted to find out whether increasing cell numbers of embryos by aggregations improves pregnancy rates after embryos transfer.

In vitro matured abattoir-derived oocytes were enucleated 22 hours after maturation by removing the zonae and bisecting the oocytes into two equal halves after incubation in medium containing 7.5 µg/ml cytochalasin and 5 µg/ml Hoeschst 33342. Cytoplasts were assessed for the presence of chromatin with fluorescence microscopy and activated by 5 min incubation in 38 µM Ca^{++}-Ionophore and 4 hour incubation in 2 mM 6-DMAP. Donor embryos were produced either in vitro (Gardner et al. 1994, Biol. Reprod. 50:390) or in vivo from superovulated cows. Five days after insemination or in vitro-fertilization, zonae of embryos were removed by pronase and embryos disaggregated in $Ca^{++}Mg^{++}$-free medium containing 5 µg/ml cytochalasin. Cytoplasts and blastomers were aligned in 0.25 M sucrose with an AC pulse of 8 volts and 500 kHZ for 5-10 sec and fused with a single DC pulse of 99.9 µsec and 625 V/cm. Fused cell pairs were cultured in modified synthetic oviduct fluid medium (SOF) supplemented with 32 mg/ml BSA st 39°C, in 7% O_2, 5% CO_2 and 88% N_2 for seven days. Embryo aggregates were produced by aggregating

three cleaved embryos (derived from the same clone) together two cays after fusion, whereas individual embryos were cultured individually throughout the culture period. Resulting morulae and blastocysts were transferred to synchronized recipients seven days after fusion. Cell numbers two days after fusion and pregnancy rates after embryo transfer, diagnosed by ultrasonography, were recorded. Because nuclear transfer embryos derived form in vitro and in vivo donor embryos were approximately equal in number, the date from both donor types was pooled.

Table 9. Embryo transfer results of nuclear transfer embryos and embryo-aggregates

Embryo	No. of recipients		No of embryos transferred	Pregnancies at Day 40	Pregnancies at Day 120	Foetuses at Day 120
Aggregate	13	34	6(46%)	7(21%)	4(31%)	4(12%)
Individual	3	10	1 (33%)	2 (20%)	0(0)	0(0)

Cell numbers of aggregates and individual embryos two days after fusion was 11.3±2.7 and 4.4±1.4, respectively. No cell number determinations were performed at blastocyst stage, as all embryos were transferred to recipients. However, from our other studies the average day 7 cell number of such individually cultured nuclear transfer blastocysts produced by single cytoplast fusion is 70±25 (range 39-127). In the case of aggregates, it is very likely that more than one clone takes part in development, therefore cell numbers could be expected to increase accordingly (Peura et al. 1997, Theriogenol. 47). Although no statistical comparisons were made due to low numbers in these preliminary experiments, embryos cultured as aggregates yielded higher ongoing pregnancy rates.

The use of embryo-derived cell lines as sources of donor nuclei in nuclear transfer may be possible in the near future. In such situations the number of donor cells may not be a limiting factor, and culturing embryo clones

as aggregates may be beneficial for achieving pregnancies.

Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

THE CLAIMS

1. A method for enucleating an oocyte which method includes:

providing an oocyte having a polar body, metaphase plate and cytoplasm;

subjecting the oocyte to a compound capable of causing attachment of the polar body to the oocyte; and

enucleating the oocyte by separating the polar body and a portion of cytoplasm containing the metaphase plate from remaining cytoplasm.

2. A method according to claim 1 wherein the oocyte contains a first polar body.

3. A method according to claim 1 or 2 wherein the oocyte is at a metaphase II arrest stage.

4. A method according to any one of claims 1 to 3 wherein the compound capable of causing attachment of the polar body to the oocyte is a compound capable of agglutinating cells.

5. A method according to claim 4 wherein the compound is a lectin.

6. A method according to claim 5 wherein the lectin is selected from the group including wheatgerm agglutinin, Concanavalin A, Canavalin A, Ricin, soybean lectin, lotus seed lectin, and phytohemaglutinin (PHA).

7. A method according to claims 5 or 6 wherein the concentration of lectin is from about 50 μg/ml to 500 μg/ml.

8. A method according to any one of claims 5 to 7 wherein the lectin is PHA at a concentration of about 200 μg/ml.

9. A method according to any one of claims 1 to 8 wherein the oocyte is subjected to the compound for a period of up to 60 minutes.

10. A method according to any one of claims 1 to 9 wherein the oocyte is further subjected to cytochalasin B at a concentration in the range of 5 to 10 μg/ml.

11. A method according to any one of claims 1 to 10 wherein the enucleation is determined by a position of the polar body to identify the metaphase plate and wherein said enucleation results in providing one portion containing the polar body and the metaphase plate and another portion containing cytoplasm

substantially free of genetic material.

12. A method for enucleating oocytes which method includes:

providing an oocyte having a polar body, metaphase plate and cytoplasm;

subjecting the oocyte to a compound capable of causing attachment of the polar body to the oocyte;

removing the zona pellucida from the oocytes to provide a zona-free oocyte containing the polar body, metaphase plate and cytoplasm; and

enucleating the zona-free oocyte by separating the polar body and a portion of cytoplasm containing the metaphase plate from remaining cytoplasm.

13. A method according to claim 12 wherein the oocyte is treated with the compound prior to removal of the zona pellucida.

14. A method according to claim 12 or 13 wherein the zona pellucida is removed by subjecting the oocytes to a pronase or protease.

15. A method according to claim 14 wherein the compound is diluted from about 1:10 to about 1:100 prior to subjecting the oocyte to a pronase or protease.

16. A cytoplast having a high cytoplasmic volume, devoid of a polar body, metaphase plate and substantially free of chromatin.

17. A cytoplast prepared by the method according to any one of claims 1 to 15.

18. A method of increasing cytoplasmic volume in an embryonic cell said method including:

providing at least two cytoplast prepared by a method of enucleating an oocyte;

providing an embryonic cell; and

fusing said cytoplasts with the embryonic cell.

19. A method according to claim 18 wherein the cytoplast is according to claim 16 or 17.

20. A method according to claim 18 or 19 wherein said cytoplasmic volume is increased by approximately 100% to approximately 150%.

21. An embryonic cell having an increased cytoplasmic volume prepared by

the method according to any one of claims 18 to 20.

22. A method for preparing a transplantation embryo, said method including:

providing a cytoplast according to claim 16 or 17;

providing a embryonic cell;

fusing said cytoplast with the embryonic cell to produce a reconstituted embryo; and

culturing the reconstituted embryo to a transferable embryo stage.

23. A method for preparing a transplantation embryo which method includes:

providing at least two cytoplast prepared by a method of enucleating an oocyte;

providing an embryonic cell;

fusing said cytoplast with the embryonic to produce a reconstituted embryo; and

culturing the reconstituted embryo to a transferable embryo stage.

24. A method according to claim 23 wherein the cytoplast is according to claim 16 or 17.

25. A transplantation embryo prepared by a method according to any one of claims 22 to 24.

26. An animal cloned from an embryo according to claim 25.

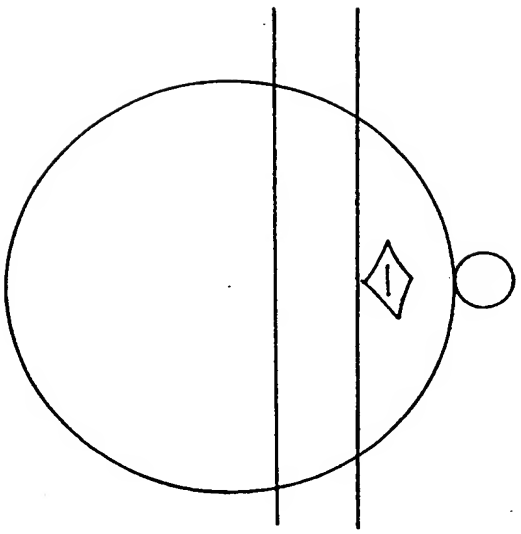

FIG 1

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00868

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 005/12; 005/16 A61D 019/00; 019/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT, Chem Abs: Keywords: Cytoplast/enucleat*; polar body; oocyte; nucle*/chromoson*; remov*/excis*

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA PLUS Keywords: oocyte; enucleat*/cytoplast; polar body; (chromosom*/ chromati*/nucle*; remov*/excis*)
BIOSIS
EMBASE
MEDLINE
CABA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	Li Meng, JJ Ely, RL Stouffer, DP Wolf. Rhesus Monkeys produced by nuclear transfer. August 1997 Biology of Reproduction 15: 454-459	All
Y	B Hogan, F Constantini, E Lacy "Manipulating the Mouse Embryo: A Laboratory Manual" Cold Srping Harbour Laboratory. USA. 1986 Pages 30-33 Section A	11-15
Y, P	AS Elsheikh, Y Takahashi, M Hishinuara, H Kanogawa. Developmental Ability of Mouse Late 2 Cell Blastomere.... February 1997. J. Vet. Med. Sc. 59(2) 107-13	11-15, 22-26
	Y Heyman, P Chesne, JP Renard. Reprogrammation Complète de noyaux embyonnaires congelès, après transfert nucléaire chez la lapin. 1990 CR Acad. Sci. Paris. Vol 311(9) 321-326	11-15, 22-26

☐ Further documents are listed in the continuation of Box C

☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
16 FEBRUARY 1998

Date of mailing of the international search report
25 FEB 1998

Name and mailing address of the ISA/AU
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION
PO BOX 200
WODEN ACT 2606
AUSTRALIA Facsimile No.: (02) 6285 3929

Authorized officer
GILLIAN ALLEN
Telephone No.: (02) 6283 2266

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00868

Box 1 Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 16, 18, 23
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The claims are too broad to make meaningful search possible.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.